

REPORT

IMPLEMENTATION AND VALIDATION OF AN ANALYTICAL METHOD FOR



**NOTOX Project 352968
NOTOX Substance 111834/B**

STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

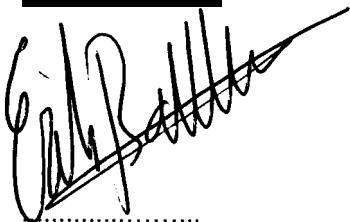
The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director

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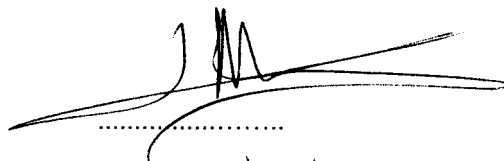


Date: 11 SEP 2002

Management



Section Head
Analytical & Physical Chemistry



Date: September 12, 2002

QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was audited by the NOTOX Quality Assurance Unit to ensure that the methods and results accurately reflect the raw data.

The dates of Quality Assurance inspections and audits are given below.
During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS/AUDITS**REPORTING DATES****on-site inspection (s)**

13-31 May 2002 (Process, Physical Chemistry)

04 June 2002

protocol inspection (s)

03 June 2002 (Study)

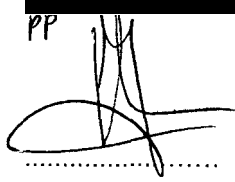
03 June 2002

report audit (s)

27 August 2002 (Study)

27 August 2002

Head of Quality Assurance

PP

..... J.T.G. Wiltse
Date: September 18, 2002

SUMMARY

Analytical method

A high performance liquid chromatographic (HPLC) method, including sample pre-concentration by solid phase extraction (SPE), for quantitative analysis of the test substance () was implemented. A Zorbax RX-C18 column was used with a gradient of acetonitrile and Milli-Q water as the mobile phase and a spectrophotometric detector set to read the absorbance at 220 nm.

A standard solution of the test substance was prepared in acetonitrile at exactly known concentrations of 13932 mg/l. In order to obtain suitable concentrations for the validation tests, these solutions were further diluted with Milli-Q water.

Several small test substance peaks were observed in chromatograms of test substance solutions. The area of two peaks, with a retention of 13.6 and 14.5 minutes, was used as test substance response in calculations during the validation tests. Peak identification was based on information supplied by the sponsor.

The HPLC method was validated for repeatability of injections, stability of the chromatographic system, stability of standard solutions, linearity and limit of detection. Additionally, the recovery of the SPE step was determined.

Repeatability of injections based on peak 1

The coefficient of variation of the response was determined to be 3.6 %, and 0.6 % for 10 replicates of a single 1.00 mg/l solution and a single 20.1 mg/l solution, respectively. Due to a relatively large coefficient of variation, it was necessary to pay extra attention to integration conditions at concentrations below 20.1 mg/l.

Repeatability of injections based on peak 2

The coefficient of variation of the response was determined to be 1.6 %, and 0.6 % for 10 replicates of a single 1.00 mg/l solution and a single 20.1 mg/l solution, respectively. Therefore it was concluded that the chromatographic and integration conditions are acceptable for further analysis in the concentration range 1.00 – 20.1 mg/l.

Stability of the chromatographic system based on both peaks

The chromatographic system was stable over at least an 80.2-hour time interval at a test substance concentration of 20.1 mg/l.

Stability of standard solutions

During Notox project 338805, standard solutions (11350 mg/l and 21460 mg/l) of the test substance in acetonitrile were determined to be stable for 24 days when stored at room temperature in the dark.

Linearity based on peak 1

A linear relationship between response and test substance concentration was found over a concentration range of 1.00 – 20.1 mg/l ($n = 7$, $r = 0.9998$).

Linearity based on [REDACTED] peak 2

A linear relationship between response and test substance concentration was found over a concentration range of 1.00 – 20.1 mg/l ($n = 7$, $r = 0.9998$).

Limit of detection based on [REDACTED] peak 1

The limit of detection for the test substance was determined to be 0.78 mg/l at an injection volume of 4000 μ l, if the standard solution was diluted with Milli-Q water.

The limit of detection for the test substance was determined to be 0.39 mg/l at an injection volume of 4000 μ l, if the standard solution was diluted with M2-medium.

The limit of detection for the test substance was determined to be 0.48 mg/l at an injection volume of 4000 μ l, if the standard solution was diluted with ISO-medium.

Limit of detection based on [REDACTED] peak 2

The limit of detection for the test substance was determined to be 0.93 mg/l at an injection volume of 4000 μ l, if the standard solution was diluted with Milli-Q water.

The limit of detection for the test substance was determined to be 0.47 mg/l at an injection volume of 4000 μ l, if the standard solution was diluted with M2-medium.

The limit of detection for the test substance was determined to be 0.60 mg/l at an injection volume of 4000 μ l, if the standard solution was diluted with ISO-medium.

Recovery

Based on peak 1, recovery at the 1.00 mg/l level was determined to be 105.5% from Milli-Q water, 112.4% from ISO-medium and 128.6% from M2-medium. Based on peak 1, recovery at the 20.1 mg/l level was determined to be 100.3% from Milli-Q water, 100.0% from ISO-medium and 99.7% from M2-medium.

Based on peak 2, recovery at the 1.00 mg/l level was determined to be 88.8% from Milli-Q water, 94.7% from ISO-medium and 97.6% from M2-medium. Based on peak 2, recovery at the 20.1 mg/l level was determined to be 99.2% from Milli-Q water, 99.5% from ISO-medium and 97.9% from M2-medium.

The high recoveries based on peak 1 at the 1.00 mg/l level from ISO-medium and particularly M2-medium were most likely caused by an interfering peak present at the position of peak 1 originating from the respective media. It was decided to accept these recovery values since when measuring actual samples in ISO- or M2-medium; calibration standards in the corresponding matrix will be prepared compensating for this effect. Moreover, if new batches of ISO- and M2-medium are prepared, the interfering peak might be eliminated.

Recoveries determined at the 20.1 mg/l level based on peak 1 and at both levels based on peak 2 were found to be acceptable.

PREFACE

Sponsor A [REDACTED]
[REDACTED] ORT
[REDACTED]

Study Monitor [REDACTED]
SHERA, Regulatory Affairs

Testing Facility NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Study Director [REDACTED]

Study plan Start: 12 June 2002
Completed: 20 June 2002

TEST SUBSTANCE

Identification [REDACTED]
Chemical name [REDACTED]
CAS RN [REDACTED]

Description Clear colourless liquid
Batch 1510-14
Purity See Certificate of Analysis
Test substance storage In refrigerator in the dark
Stability under storage conditions Stable
Expiry date 01 January 2003

The sponsor is responsible for all test substance data unless determined by NOTOX.

Note: Don't heat up the test substance above 50°C

PURPOSE AND PRINCIPLE

The purpose of the study was to develop and validate a method for the quantitative analysis of [REDACTED]. A high performance liquid chromatographic (HPLC) method, including sample pre-concentration by solid phase extraction (SPE), was implemented. The method was validated for repeatability of injections, stability of the chromatographic system, linearity and limit of detection. Additionally, the recovery of the SPE step was determined.

ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years:
protocol, report, test substance reference sample and raw data.

REAGENTS

Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore, Bedford, MA, USA														
Acetonitrile	HPLC-grade, Labscan, Dublin, Ireland														
ISO-medium	Milli-Ro water containing the following components: <table> <tr> <td>Ca²⁺</td><td>80 mg/l</td></tr> <tr> <td>Mg²⁺</td><td>12 mg/l</td></tr> <tr> <td>Na⁺</td><td>15 mg/l</td></tr> <tr> <td>K⁺</td><td>3 mg/l</td></tr> <tr> <td>Cl⁻</td><td>145 mg/l</td></tr> <tr> <td>SO₄²⁻</td><td>49 mg/l</td></tr> <tr> <td>HCO₃⁻</td><td>47 mg/l</td></tr> </table>	Ca ²⁺	80 mg/l	Mg ²⁺	12 mg/l	Na ⁺	15 mg/l	K ⁺	3 mg/l	Cl ⁻	145 mg/l	SO ₄ ²⁻	49 mg/l	HCO ₃ ⁻	47 mg/l
Ca ²⁺	80 mg/l														
Mg ²⁺	12 mg/l														
Na ⁺	15 mg/l														
K ⁺	3 mg/l														
Cl ⁻	145 mg/l														
SO ₄ ²⁻	49 mg/l														
HCO ₃ ⁻	47 mg/l														
M2-medium	Medium formulated according to ISO/IS 8692														

ANALYTICAL METHOD

A high performance liquid chromatographic (HPLC) method for quantitative analysis of the test substance was implemented by NOTOX B.V. A schematic lay-out of the instrumentation used is illustrated in Figure 1. Using an autosampler, samples were injected onto an SPE column (Pre column) using a 1.5 ml/min flow of Milli-Q water generated by pump 2. After 5 minutes, the valve is switched and the SPE column is desorbed by the mobile phase gradient generated by pump 1. The conditions of the method are described below:

Column	Zorbax Rx-C18, 250 x 4.6 mm; d _p =5 µm (Analytical column was supplied by the sponsor)		
SPE Column	PLRP-S, 2 x 20 mm (SPE column was supplied by the sponsor)		
Column temperature	25°C		
Mobile phase A	Acetonitrile		
Mobile phase B	Milli-Q water		
Gradient program (pump 1)	Time (min.)	%A	%B
	0	46	54
	5	46	54
	10	100	0
	13	100	0
	14	46	54
	19	46	54
Flow	2 ml/min		
Detection wavelength	220 nm		
Injection volume	4000 µl		

PREPARATION OF SOLUTIONS

A standard solution of the test substance was prepared in acetonitrile at an exactly known concentration of 13932 mg/l. Suitable concentrations for the validation tests were obtained by dilution of this solution with Milli-Q water.

VALIDATION OF THE ANALYTICAL METHOD

The HPLC method was validated for repeatability of injections, stability of the chromatographic system, linearity and limit of detection. Additionally, the recovery of the SPE step was determined.

Repeatability of injections

Each of two solutions (1.00 mg/l and 20.1 mg/l) was injected in tenfold into the HPLC system and the responses were recorded. The coefficient of variation of the response was calculated for each concentration.

Stability of the chromatographic system

A 20.1 mg/l solution was injected (in duplicate) several times in an 80.2-hour time interval. The maximum deviation of the response was calculated.

Stability of standard solutions

Stability of standard solutions was determined during Notox project 338805.

Linearity

From one standard solution (13932 mg/l), seven dilutions were prepared. This resulted in a concentration range of 1.00 – 20.1 mg/l. Each of the solutions was injected in duplicate. Responses were plotted against concentrations. A linear regression program was used to calculate the regression line from the responses and concentrations.

Limit of detection

A 0.497 mg/l solution was injected in duplicate. In each chromatogram, the height (expressed in mV) of the test substance peak was measured as well as the noise level of the system (mV). The limit of detection was calculated from the mean peak height and the mean noise level.

Recovery

Using a 13932 mg/l standard solution, blank Milli-Q water, blank M2-medium and blank ISO-medium were spiked at levels of 1.00 and 20.1 mg/l. These samples were analysed with the method described under 'Analytical Method'.

Using a 13932 mg/l standard solution, calibration solutions in Milli-Q water, M2-medium and ISO-medium of 40.4 and 808 mg/l were prepared. These samples were analysed using 100 µl loop-type injection. For these injections, the set-up as illustrated in Figure 1 was modified with an additional (manual) 6-port valve with 100 µl injection loop inserted between the 6-port valve shown in Figure 1 and the analytical column.

Recovery of the SPE procedure was determined by comparison of the results obtained with 4000 µl injection of the 1.00 and 20.1 mg/l samples and 100 µl injection of the 40.4 and 808 mg/l samples.

DATA HANDLING

Response (R) Peak area of the test substance [units]

Response factor Response/concentration [units* l * 10^3 /g]

Mean
$$\bar{x} = \frac{1}{n} \sum_{i=1}^n x_i$$

where:

x_i : measured value

n : number of measurements

Standard deviation
$$s_{n-1} = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{(n-1)}}$$

Coefficient of variation (standard deviation/mean) * 100%

Maximum deviation [(highest - lowest)/mean] * 100%
where 'mean' is the mean value of the highest and the lowest value.

Linearity A linear regression program was used to calculate the regression line from the responses and concentrations. Linear regression analysis was performed using the least squares method.

Regression line: $Y = aX + b$

where:

Y : response

X : concentration

a : slope

b : intercept

Correlation coefficient During regression analysis, the correlation coefficient (r) was calculated.

Limit of detection The limit of detection is defined as the lowest concentration of test substance that can be distinguished from instrumental noise using the analytical method described.

Limit of detection = $((3 * \text{noise level}) / \text{signal}) * \text{conc.}$

where:

noise level (N) : height of the noise [mV]

signal (S) : height of the test substance peak [mV]

conc. : concentration of test substance [mg/l]

Recovery

$$\frac{R_{SPE-HPLC}}{R_{Direct}} * \frac{C_{Direct}}{C_{SPE-HPLC}} * \frac{V_{Direct}}{V_{SPE-HPLC}} * 100\%$$

where:

$R_{SPE-HPLC}$: Response of sample analysed by SPE-HPLC [units]
R_{Direct}	: Response of sample analysed by 100 μ l direct injection [units]
$C_{SPE-HPLC}$: Concentration of sample analysed by SPE-HPLC [mg/l]
C_{Direct}	: Concentration of sample analysed by 100 μ l direct injection [mg/l]
$V_{SPE-HPLC}$: Volume of sample analysed by SPE-HPLC [ml]
V_{Direct}	: Volume of sample analysed by 100 μ l direct injection [ml]

RESULTS

A high performance liquid chromatographic (HPLC) method was implemented for the quantitative analysis of [REDACTED]. The HPLC method was validated for repeatability of injections, stability of the chromatographic system, stability of standard solutions, linearity and limit of detection. Additionally, the recovery of the SPE step was determined.

HPLC chromatograms of the test substance and of a blank solution (Milli-Q water) are shown in Figures 2 and 3, respectively. Several small test substance peaks were observed in the chromatograms of test substance solutions, as is illustrated in Figure 2. The area of two peaks, with retention times of 13.6 and 14.5 minutes, were used as test substance response in calculations during the validation tests. Peak identification was based on information supplied by the sponsor.

The calculations for the validation tests were performed using not-rounded concentrations and responses. Therefore, some differences might be observed when calculating the statistical parameters using the values as mentioned in the tables.

Repeatability of injections

The results are summarised in Tables 1 and 2.

Table 1 Repeatability of injections based on [REDACTED] peak 1.

Concentration [mg/l]	Response ¹ [units]	Standard deviation [units]	Coefficient of variation [%]
1.00	4839	176	3.6
20.1	87178	496	0.6

¹ Mean of 10 replicates of a single solution.

From these results, it was concluded that the chromatographic and integration conditions are acceptable for further analysis at the concentration level of 20.1 mg/l. Due to a relatively large coefficient of variation, it was necessary to pay extra attention to integration conditions at concentrations below 20.1 mg/l.

Table 2 Repeatability of injections based on [REDACTED] peak 2.

Concentration [mg/l]	Response ¹ [units]	Standard deviation [units]	Coefficient of variation [%]
1.00	3517	57	1.6
20.1	76215	457	0.6

² Mean of 10 replicates of a single solution.

From these results, it was concluded that the chromatographic and integration conditions are acceptable for further analysis in the concentration range 1.00 – 20.1 mg/l.

Stability of the chromatographic system

Tables 3 and 4 show the responses for the first duplicate injection, the duplicate injection with the lowest response, the duplicate injection with the highest response and the last duplicate injection.

Table 3 Stability of the chromatographic system based on [REDACTED] peak 1.

Elapsed time [hours]	Response ¹ [units]	Maximum deviation [%]
0.0	87482	3.7
0.7	88997	
57.3	87175	
58.0	85728	
79.5	86622	
80.2	87880	

¹ For a 20.1 mg/l solution.

From these results, it was concluded that the chromatographic system was stable over at least an 80.2-hour time interval at the concentration tested (20.1 mg/l).

Table 4 Stability of the chromatographic system based on [REDACTED] peak 2.

Elapsed time [hours]	Response ¹ [units]	Maximum deviation [%]
0.0	77175	5.3
0.7	78596	
57.3	75804	
58.0	74573	
79.5	75323	
80.2	76314	

¹ For a 20.1 mg/l solution.

From these results, it was concluded that the chromatographic system was stable over at least an 80.2-hour time interval at the concentration tested (20.1 mg/l).

Stability of standard solutions

During Notox project 338805, standard solutions (11350 mg/l and 21460 mg/l) of the test substance in acetonitrile were determined to be stable for 24 days when stored at room temperature in the dark.

Linearity

The results are summarised in Tables 5 and 6, the corresponding regression lines are shown in Figure 4 and 5.

Table 5 Linearity based on [REDACTED] peak 1.

Concentration [mg/l]	Response ¹ [units]
1.00	4900 / 5227 ²
2.51	11345 / 11565
5.02	21885 / 21649
7.52	32217 / 32685
10.0	44037 / 43306
15.0	63665 / 64754
20.1	87359 / 87499
Slope	4.31×10^3
Intercept with Y-axis	3.08×10^2
Weighting factor	None
r	0.9998

¹ Duplicate measurements.

² This injection has considered to be an outlier.

From these results, it was concluded that there is a linear relationship between response and concentration in the concentration range of 1.00 – 20.1 mg/l.

Table 6 Linearity based on [REDACTED] peak 2.

Concentration [mg/l]	Response ¹ [units]
1.00	3619 / 3480
2.51	9228 / 9390
5.02	18743 / 18257
7.52	27828 / 28005
10.0	37482 / 37277
15.0	55103 / 56640
20.1	76405 / 75824
Slope	3.79×10^3
Intercept with Y-axis	-4.23×10^2
Weighting factor	None
r	0.9998

¹ Duplicate measurements.

From these results, it was concluded that there is a linear relationship between response and concentration in the concentration range of 1.00 – 20.1 mg/l.

Limit of detection

From two chromatograms of a 0.497 mg/l solution of the test substance diluted in Milli-Q water, the mean noise level (N) was determined to be 0.2 mV. From the same chromatograms, the test substance signal (S), i.e. the mean height of the test substance peak, was determined to be 0.38 mV for [REDACTED] 0.93 mg/l for [REDACTED] peak 2 at an injection volume of 4000 µl.

From two chromatograms of a 0.497 mg/l solution of the test substance diluted in M2-medium, the mean noise level (N) was determined to be 0.1 mV. From the same chromatograms, the test substance signal (S), i.e. the mean height of the test substance peak, was determined to be 0.39 mV for M [REDACTED] calculated to be 0.39 mg/l for [REDACTED] peak 1 and 0.47 mg/l for [REDACTED] peak 2 at an injection volume of 4000 µl.

From two chromatograms of a 0.497 mg/l solution of the test substance diluted in ISO-medium, the mean noise level (N) was determined to be 0.1 mV. From the same chromatograms, the test substance signal (S), i.e. the mean height of the test substance peak, was determined to be 0.40 mV for [REDACTED] 48 mg/l for [REDACTED] peak 1 and 0.60 mg/l for [REDACTED] peak 2 at an injection volume of 4000 µl.

Recovery

Based on peak 1, recovery at the 1.00 mg/l level was determined to be 105.5% from Milli-Q water, 112.4% from ISO-medium and 128.6% from M2-medium. Based on peak 1, recovery at the 20.1 mg/l level was determined to be 100.3% from Milli-Q water, 100.0% from ISO-medium and 99.7% from M2-medium.

Based on peak 2, recovery at the 1.00 mg/l level was determined to be 88.8% from Milli-Q water, 94.7% from ISO-medium and 97.6% from M2-medium. Based on peak 2, recovery at the 20.1 mg/l level was determined to be 99.2% from Milli-Q water, 99.5% from ISO-medium and 97.9% from M2-medium.

The high recoveries based on peak 1 at the 1.00 mg/l level from ISO-medium and particularly M2-medium were most likely caused by an interfering peak present at the position of peak 1 originating from the respective media. It was decided to accept these recovery values since when measuring actual samples in ISO- or M2-medium; calibration standards in the corresponding matrix will be prepared compensating for this effect. Moreover, if new batches of ISO- and M2-medium are prepared, the interfering peak might be eliminated.

Recoveries determined at the 20.1 mg/l level based on peak 1 and at both levels based on peak 2 were found to be acceptable.

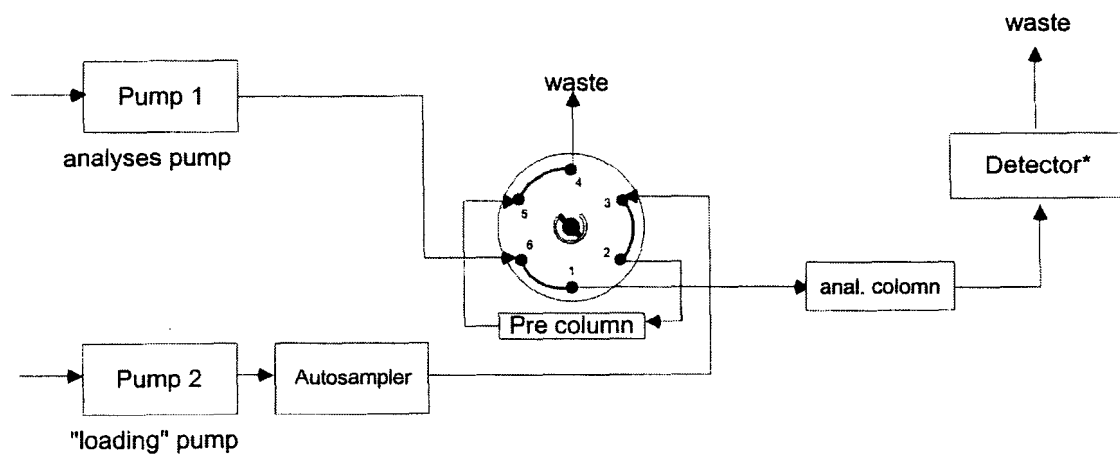


Figure 1 Schematic lay-out of the instrumentation used.

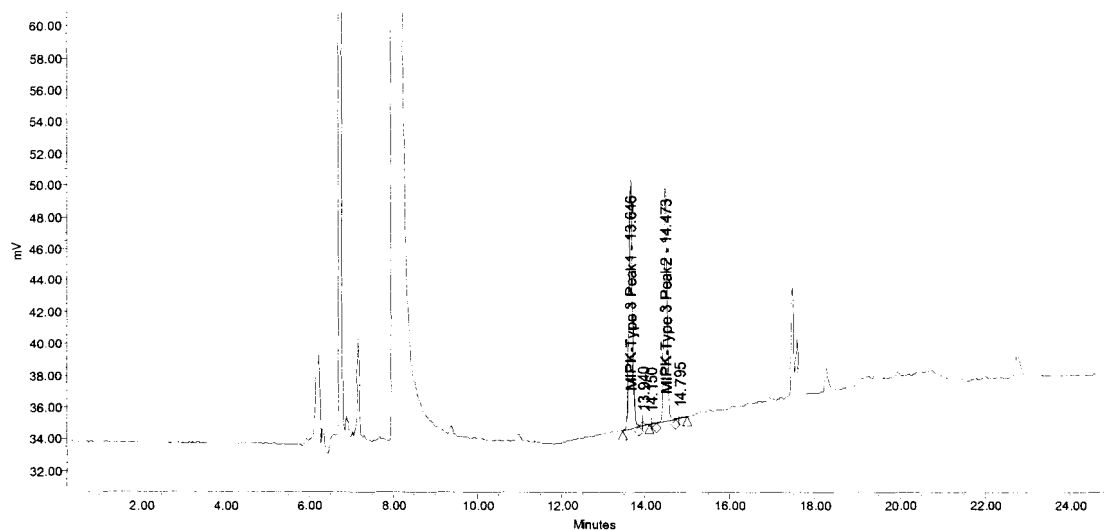


Figure 2 HPLC chromatogram of a 20.1 mg/l solution of [redacted] in Milli-Q water [res.id. 1468].

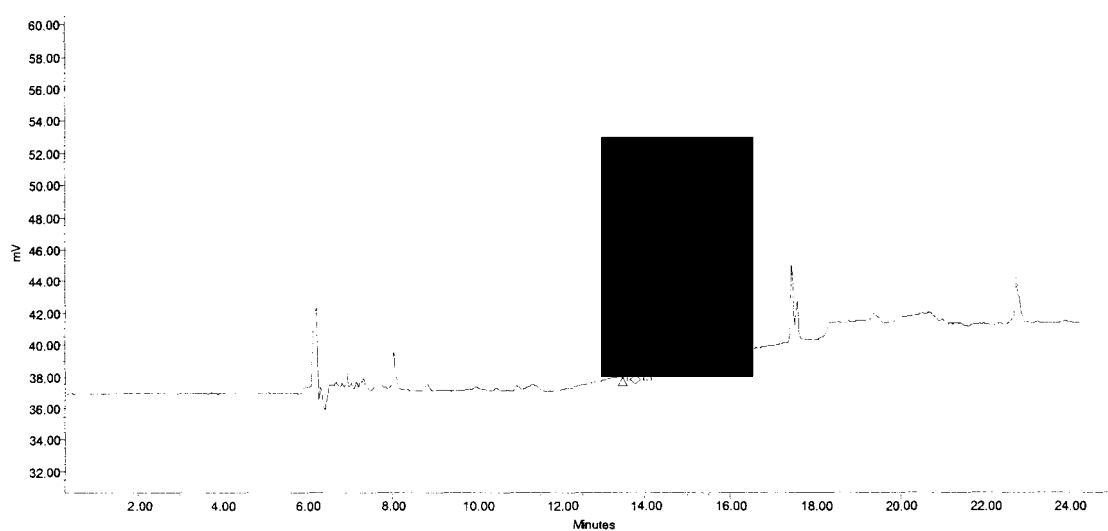


Figure 3 HPLC chromatogram of a blank solution (Milli-Q water) [res.id. 1474].

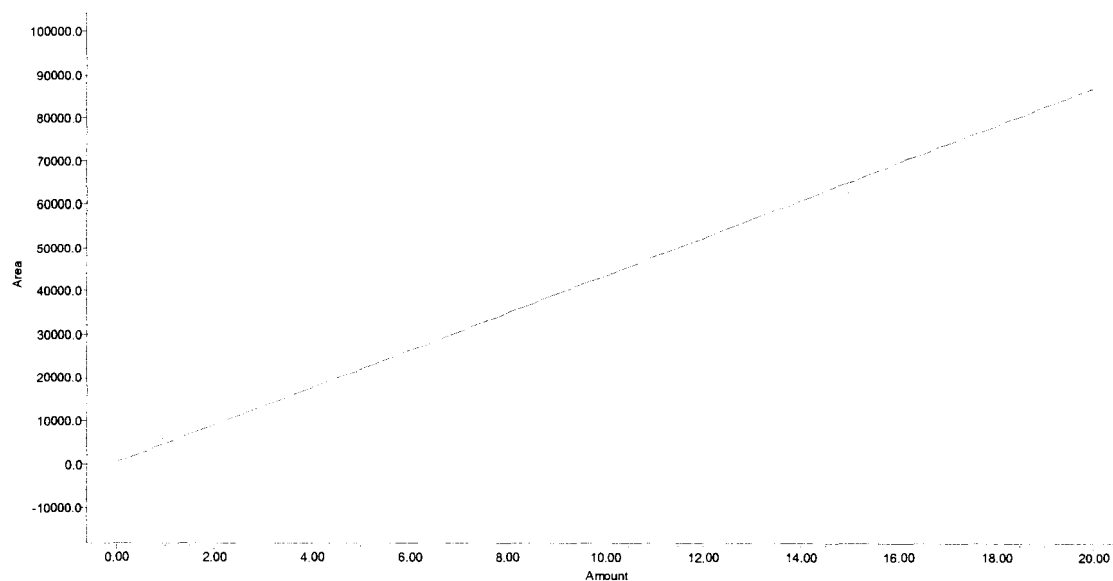


Figure 4 Regression line: response of [redacted] k 1 as a function of concentration [cal.curve id. 1472].

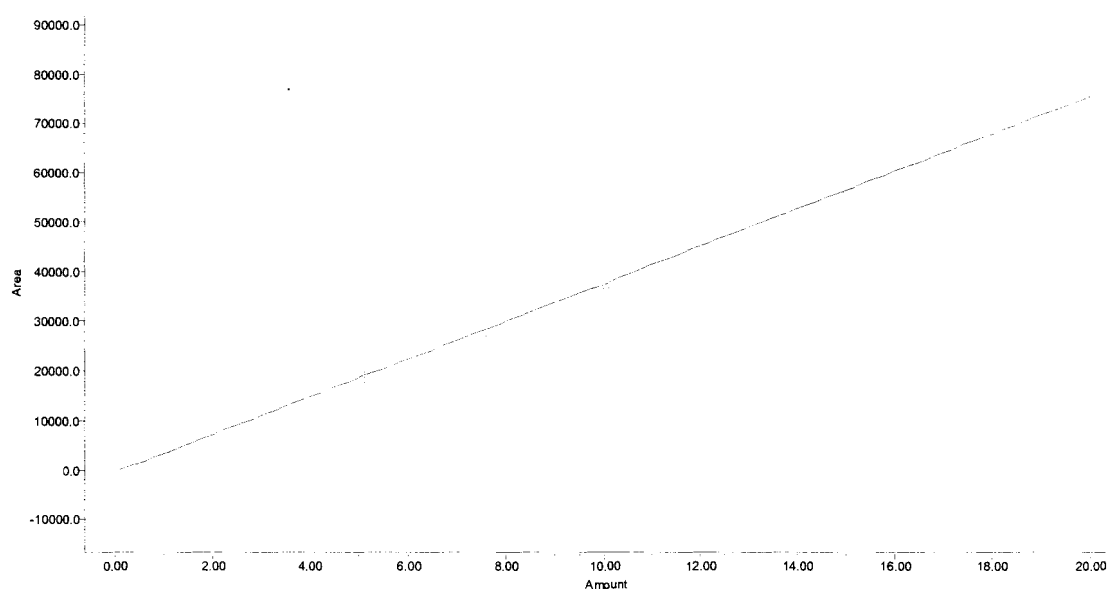


Figure 5 Regression line: response of [redacted] as a function of concentration [cal.curve id. 1473].